Reconstructive Urology

Protective Effect of Wharton's Jelly-derived Mesenchymal Stem Cells on Renal Fibrosis in Rats with Unilateral Ureteral Obstruction

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Article info

Abstract

Background: Renal failure is a global medical problem. The use of mesenchymal stem cells (MSCs) for preservation and regeneration of renal tissue in acute and chronic kidney diseases has recently been the focus of investigation.

Objective: To evaluate the protective effect of MSC injections in a rat model of kidney obstruction.

Design, setting, and participants: We assigned 15 male Wistar rats to three separate groups: the normal group underwent left nephrectomy; the control group underwent laparotomy and left ureter ligation followed by saline injection into the aorta; and the study group received MSCs injected into the aorta inferior to the left renal artery after ligation of the left ureter. Kidneys were harvested 4 wk later and renal parenchyma samples were used for trichrome staining and for expression analyses.

Outcome measurements and statistical analysis: The degree of kidney fibrosis was assessed on pathology. Real-time polymerase chain reaction was used to determine expression levels of VEGF, TNF-α, and E-cadherin, and ΔCT and ΔΔCT values were calculated. Data were analyzed using SPSS v19 with paired *t* tests and nonparametric independent-sample Kruskal-Wallis tests.

Results and limitations: Fibrosis in the study group decreased from grade 3 or 4 to grade 1. In the control group, TNF-α expression increased and E-cadherin expression decreased. After MSC injection into obstructed kidneys, TNF-α and E-cadherin expression levels decreased and increased respectively, reaching similar levels to those in the normal group. No correlation between tissue regeneration and VEGF levels was observed. More research is needed to focus on other angiogenic factors.

Conclusions: MSC injection could prevent fibrosis in obstructed rat kidney via alterations in TNF-α and E-cadherin expression.

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Patient summary: We investigated the effect of stem cell injection in rats with kidney obstruction. The treatment led to changes in the levels of two biomarkers and reduced the amount of kidney fibrosis caused by kidney obstruction.

1. Introduction

Renal failure is a common health problem worldwide [1] and is categorized as acute renal failure (ARF) or chronic renal failure (CRF). ARF accounts for approximately 19.2 out of every 1000 hospitalizations and has high morbidity and mortality. Its prevalence among patients admitted to intensive care units after vessel and abdominal surgery is nearly 20% [2]. In 2017, 1.2 million individuals died due to chronic kidney disease (CKD), contributing disability-adjusted life-years (DALYs) of 35.8 million [3]. In Iran, the most common cause of ARF is glomerulonephritis, followed by diabetes mellitus and hypertension [4]. The most common causes of CRF globally are diabetes mellitus, hypertension, glomerulonephritis, and urologic diseases [5]. Common treatments for CRF include peritoneal dialysis, hemodialysis, and kidney transplantation, each of which has its own side effects and problems [6]. Therefore, recent efforts have focused on new treatment methods that could lead to return of kidney function, decrease morbidities and mortality, and even reduce costs [7]. In the present study we investigated the role of the inflammatory cytokines TNF-α and VEGF and the adhesion molecule E-cadherin in renal fibrosis caused by ureteral obstruction, which leads to renal failure. We also evaluated the effects of mesenchymal stem cells (MSCs) from umbilical-cord Wharton's jelly in preventing ureteral obstruction–induced renal fibrosis and the expression of inflammatory cytokines.

2. Materials and methods

The study protocol was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences. Anesthesia was in accordance with the institutional animal care protocol (ketamine 75 mg/kg and xylazine 15 mg/kg injected intraperitoneally).

2.1. Animals

Fifteen male Wistar rats, weighing between 250 and 300 g, were randomly divided into three groups (n = 5 per group). Male rats were used to prevent any potential effects of hormonal fluctuation on the results.

2.2. Procedure

The study was carried out from September 2015 to March 2018. Rats in the normal group underwent left nephrectomy and a portion of the renal parenchyma was fixed in formalin for pathology examination. Another specimen from the kidney was kept at −80 °C for expression analyses. Rats in the control group underwent midline laparotomy and the left

ureter was identified in the retroperitoneal space and was closed with 4-0 silk sutures. Simultaneously, normal saline was injected into the aorta inferior to the renal artery while pressing the distal part of aorta with a finger and the abdomen was closed with 3-0 Vicryl sutures. Rats in the study group underwent ligation of the left ureter as for the control group, except 10^6 MSCs were injected into the aorta inferior to the renal artery instead of saline. After recovery from anesthesia, rats in the control and study groups were sent to the animal center. After 4 wk (approx. 25–30 d), the animals were sacrificed and their left kidneys were removed. Separate kidney samples were fixed in formalin for pathology evaluation and frozen at −80 °C for expression analyses.

2.3. MSC extraction from umbilical cord

Umbilical cords from neonates delivered in hospitals affiliated to Jundishapur University were separated under completely sterile conditions and sent to the Cellular and Molecular Survey Center. The neonates were born to primigravid women who were seronegative for human immunodeficiency virus, hepatitis B virus, and hepatitis C virus, with no underlying disease, without any past operations, and with no previous history of transfusion of blood and/or blood products. MSCs were separated via an enzymatic method by slicing and using collagenase and hyaluronidase enzymes. During culture in growth medium containing nutrients, stem cells started to proliferate and fully expand over the culture medium. The cells were separated and transferred to a second culture (passage). We used passage 2 cells because research has shown that passage 2 cells do not present HLA-2 and do not have issues caused by immunogenicity. To confirm that the cultured cells were indeed pluripotent stem cells, we directed them towards differentiation to specialized cells (bone, cartilage, and muscle) and demonstrated the differentiation using tissue-specific staining [8–12].

2.4. Pathology

Formalin-fixed samples were sent to the pathology laboratory and were stained with trichrome. One pathologist evaluated the slides to determine the degree of fibrosis.

2.5. Gene expression analyses

A parenchyma sample from each kidney was powdered using liquid nitrogen under sterile and RNase-free conditions. RNA was extracted using an RNase Plus Mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instruction. RLT buffer (600 μl), 70% ethanol, RW1 buffer (700 μl), RPE buffer (500 μl), and 50 μl of RNase-free water were added. The RNA extracts can be kept for 1 yr at −20 °C to −70 °C. Tubes were labeled as normal, control, and study groups.

To convert RNA to cDNA, a RevertAid First-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) was used. GAPDH primers were used for the positive control and for conducting real-time quantitative polymerase chain reaction PCR (qPCR).

Real-time PCR was carried out to amplify DNA using primers for GAPDH, VEGF, TNF-α, and E-cadherin with a SYBR Green PCR Master Mix

kit. Amplifications were carried out in triplicate to ensure reliability, with an extra tube as a negative control.

2.6. Data analysis

Pathology slides were reviewed by a pathologist. Results obtained from qPCR, namely the cycle threshold (CT), melt curve, and amplification curves, were analyzed with SPSS v19 using paired t tests and nonparametric independent-sample Kruskal-Wallis tests.

3. Results

3.1. Macroscopic results

Figure 1 shows the macroscopic kidney appearance for the three groups. Gross morphologic differences between the groups are markedly visible.

3.2. Microscopy results

Pathology slides were evaluated and scored on a scale from 0 to 4 according to the degree of fibrosis, where 0 represents no fibrosis and 4 denotes very extensive fibrosis. As expected, no fibrosis was observed for the normal group, whereas the degree of fibrosis was scored as 3 or 4 in the control group and 1 in the study group (Fig. 2).

3.3. qPCR results

ΔCT values for VEGF, TNF-α, and E-cadherin were calculated with the 95% confidence interval using the formula $\Delta CT = CT_X - CT_{GAPDH}$, where X denotes VEGF, TNF-α, or E-cadherin.

We used the Pfaffl formula to calculate $2^{-\Delta\Delta CT}$ for between-group comparisons. The closer the result is to 1, the smaller is the difference between the two groups. For TNF-α, $2^{-\Delta\Delta CT}$ was 1.93 between the control and normal groups, and 0.62 between the study and normal groups. For E-cadherin, $2^{-\Delta\Delta CT}$ was 0.433 between the control and normal groups, and 0.863 between the study and normal groups. For VEGF, $2^{-\Delta\Delta CT}$ was 1.071 between the control and normal groups and 0.909 between the study and normal groups. The $2^{-\Delta\Delta CT}$ values for the group comparisons are shown in Figure 3.

4. Discussion

Urinary obstruction is one of the most common causes of renal failure and its treatment requires timely diagnosis and removal of the obstruction [4]. MSC therapy is a new and promising strategy in treating renal failure, whether acute or chronic. One of the reasons for the effect of MSCs is the mesenchymal origin of nephrons and tubules. MSCs can be isolated from different organs provided they have low immunogenicity. In the present study, we used MSCs obtained from Wharton's jelly of umbilical cord which has low immunologic effect [13,14]. One of the qualities of mesenchymal stem cells is their ability of gathering in damaged and swollen tissues. Intravascular injection means that MSCs can migrate to glomerules, tubules, periglomerular vessels, and interstitial tissues, whether in acute or chronic conditions [10] This can also be achieved via intravenous injection, as demonstrated by Huang et al. [15]. It is noteworthy that this migration is mediated by inflammatory cytokines [16].

Fig. 1 – Macroscopic evaluation of kidneys: 1, normal kidney; 2, obstructed kidney; and 3, obstructed kidney after mesenchymal stem cell injection.
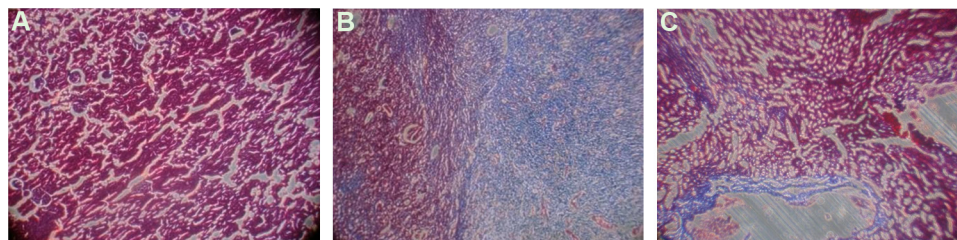

Fig. 2 – Microscopic appearance of (A) normal rat kidney; (B) obstructed rat kidney; and (C) obstructed rat kidney after mesenchymal stem cell injection.
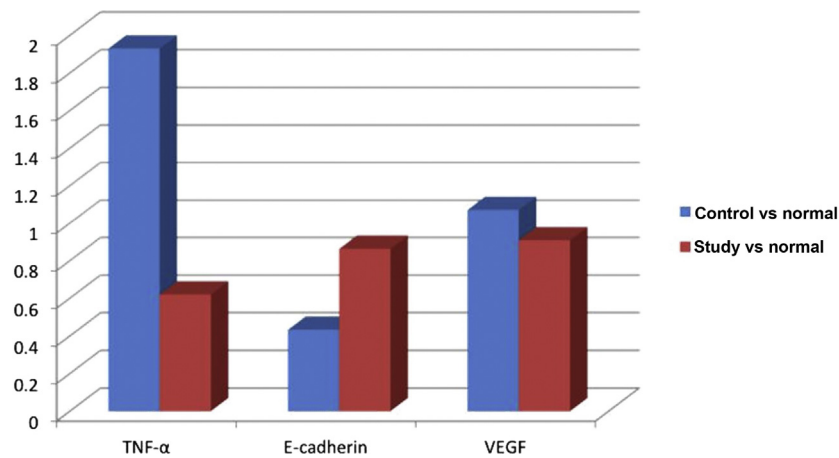
Fig. 3 – Between-group comparisons (control vs normal and test vs normal) of $2^{-\Delta\Delta CT}$ values for TNF-α, E-cadherin, and VEGF expression.

Studies have shown that stress resulting from urinary obstruction causes a number of changes in inflammatory mediators in kidney. TNF-α is a cytokine that has a role in mediating renal damage; it increases other inflammatory cytokines and extracellular matrix and causes fibrosis [7,17]. VEGF plays a role in matrix remodeling, chemotaxis of monocytes, expression of adhesive molecules, and proliferation of peritubular capillaries, which are necessary for tubular regeneration. Other inflammatory cells that play a role in inflammatory nephropathy include HGF-1, IGF-1, and FGF. E-Cadherin, which is a marker for normal epithelium, decreases in obstructed kidneys [4]. Microscopy revealed that after 48 hr of obstruction, the ureter and pelvis become loose, the renal papilla starts to blunt, and the parenchyma starts to thin. After 12 d, the cortex shallows slightly and pelvicalyceal dilatation continues. After 21–28 d, the kidney returns to its original size but the cortex and medulla become thinner. Finally, 6 wk after ureteral obstruction, the kidney becomes larger and cystic and has a lower weight than the contralateral kidney; nevertheless, it is of note that when the obstruction is incomplete these changes are not as apparent. Interstitial edema develops 48 h after obstruction, and after 7 d severe edema is apparent, with widening of the Bowman space and cellular hyalinization. After 12 d, papillary necrosis, focal tubular destruction, and inflammatory cellular responses occur, with interstitial fibrosis after 16 d. Massive glomerular destruction, tubular atrophy, interstitial fibrosis, and connective tissue proliferation in the collecting system are evident in humans after 5 wk [18].

As shown in Figure 1, renal parenchyma in the control group became thinner, the collecting system widened, and the parenchyma became pale; it can be said that the kidney has essentially become hydronephrotic. Parenchyma in the study group was slightly paler and the collecting system was slightly looser than in the normal group. In comparison to the control group, renal parenchyma in the study group was preserved and there was much less dilatation of the collecting system, demonstrating the protective effect of the MSCs.

There was no kidney fibrosis in the normal group, whereas a fibrosis score of 3 to 4 was assigned for interstitial tissues in obstructed kidneys. This confirms that ureteral obstruction causes deposition of collagen in renal tissue and leads to fibrosis of interstitial tissue. Between-group comparisons revealed that injection of MSCs led to a decrease in the degree of fibrosis in obstructed kidneys, resulting in a score of 1. However, it should be noted that despite this reduction, there was still a certain degree of fibrosis in comparison to the normal group (Fig. 2A–C).

To investigate the effect of ureteral obstruction on renal fibrosis, cytokines, and inflammatory mediators, and whether MSC injection could offer protection against these changes, we measured expression levels of VEGF, TNFα, and E-cadherin in kidney via qPCR. We evaluated the significance of differences between the three groups using CT and $2^{-\Delta\Delta CT}$ values, applying Kruskal-Wallis and t tests.

Between-group comparisons of E-cadherin levels using the $2^{-\Delta\Delta CT}$ method revealed a value of 0.433 for the control versus the normal group, with a significant difference in expression ($p = 0.001$). The value for the study versus the normal group was 0.863, with no significant difference in expression ($p = 0.191$). The E-cadherin levels significantly differed between the study and control groups ($p = 0.000$). We can conclude that in a kidney with ureteral obstruction, E-cadherin, which reflects the connection between cells of the interstitial tissue, decreases, but MSC injection leads to an increase in E-cadherin expression, reaching the level observed in normal kidney parenchyma.

Docherty et al. [19] showed that although it had been reported that E-cadherin decreased in kidneys with obstructive damage, they found that E-cadherin mRNA increased in a kidney with a ligated ureter in both acute and chronic phases, which puts into question the role of epithelial growth factor. Comparison of our results with the findings reported by Docherty et al. highlights a challenge in elucidating the role of E-cadherin in the pathogenesis of obstructive renal failure.

Between-group comparison of VEGF levels using the $2^{-\Delta\Delta CT}$ method revealed significant differences for study

versus normal ($p = 0.001$) and study versus control (-$p = 0.000$) groups, but not for the normal group versus the control group ($p = 0.256$). This indicates a lack of meaningful difference in VEGF expression between obstructed and normal kidneys, but that MSC injection in the study group decreased the level of VEGF in comparison to the other two groups. As VEGF has a key role as an angiogenic factor, this finding requires further investigation. On the basis of our results, we are unable to conclude whether VEGF has any role in kidney remodeling after obstruction and why its level in obstructed kidney is similar to that in normal kidney. It is also unclear why MSC injection induced a decrease in VEGF expression rather than a decrease; this shows that we have yet to fully understand the role of VEGF in kidney obstruction and remodeling.

Comparison of TNF-α levels using the $2^{-\Delta\Delta CT}$ method revealed a value of 1.93 for the control group versus the normal group ($p = 0.022$), indication a significant difference in TNF-α levels between these groups and higher TNF-α expression in the control group. The value of 0.62 for the study group versus the normal group ($p = 0.142$) demonstrates that TNF-α levels did not significantly differ between these groups. The difference in TNF-α between the study and control groups was significant ($p = 0.001$). We can conclude that the increase in TNF-α expression in kidney due to an obstructed ureter can be reversed by MSC injection to reach a level similar to that in normal kidney parenchyma. Between-group t-test results for comparisons for E-cadherin, TNF-α, and VEGF are shown in Table 1.

Using stem cells extracted from human femur and tibia, Qian et al. [8] observed tissue regeneration after renal failure induced by nephrotoxins. The authors reported pathology results similar to our findings, but they used CK18 and AQP1 as epithelial proliferation markers to investigate tissue regeneration rather than TNF-α, E-cadherin, and VEGF.

Verdoorn et al. [6] measured bioactive pattern of lipids to evaluate tissue regeneration in rats whose kidneys were damaged via unilateral ureteral obstruction, but they did not investigate TNF-α, VEGF, and E-cadherin levels. Nevertheless, the lipid patterns demonstrated the effect of stem cells in tissue regeneration after ureteral obstruction [6].

Two separate studies investigated injection of MSCs in rats with CKD, which is similar to Allport's syndrome in humans [9,20]. Both groups came to the conclusion that MSCs prevent progression of interstitial fibrosis and glomerular sclerosis, but their role in recovery of kidney function in rats was not confirmed. Results from the present study are congruent with these findings and show the role of stem cells in tissue regeneration. However, these studies investigated CKD resulting from glomerular disease and did not consider obstructed nephropathy as one of the causes of CKD [9,20].

Semedo et al. [21] investigated the role of stem cells in improving kidney function following acute kidney injury (AKI) and CKD by evaluating TNF-α, α-SMA, FSP-1, TGF-β, and IL-6. Similar to our study, the authors found that TNF-α has an important role in fibrotic renal failure and that stem cells help in retaining renal function by decreasing TNF-α. However, Semedo et al. investigated non-obstructed AKI and CKD, while we examined AKI due to obstructive uropathy. Liu et al. [22] also found that MSCs can reduce α-SMA, TGF-β, and TNF-α, in addition to their ability to decrease malondialdehyde, reactive oxygen species, and glutathione rises induced by unilateral ureteral obstruction. The same group found that human MSC injection reduced not only inflammatory factors but also inflammatory cell infiltration and extracellular matrix deposition [23].

Our study showed that MSCs can have a protective effect on the kidney if they are injected at the beginning of acute renal obstruction. Their role in chronic ureteral obstruction may be different, so further research is required to determine whether this is the case. Considering the lack of satisfactory response of VEGF expression to MSC injection, investigation of the response of other angiogenic factors would be beneficial.

5.   Conclusions

MSC injection could prevent fibrosis in rats with unilateral ureteral obstruction, in line with the changes in TNF-α and E-cadherin expression observed. No effect on VEGF was observed with regard to tissue repair in renal obstruction.

Table 1 – Between-group t-test results for comparisons for E-cadherin, TNF-, and VEGF

	Mean	Number	Standard deviation	p value
E-Cadherin				
Normal	2.310	5	0.30402	0.191
Test	2.542	5		
Normal	2.310	5	0.27115	0.001
Control	3.522	5		
Test	2.524	5	0.18913	0.000
Control	3.522	5		
TNF-α				
Normal	1.456	5	0.84097	0.142
Test	2.144	5		
Normal	1.456	5	0.58491	0.022
Control	0.506	5		
Test	2.144	5	0.42020	0.001
Control	0.506	5		
VEGF				
Normal	2.196	5	0.10770	0.001
Test	2.636	5		
Normal	2.196	5	0.16882	0.256
Control	2.096	5		
Test	2.636	5	0.11158	0.000
Control	2.096	5		

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